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(54) Title: AGGLUTINATION TEST

(57) Abstract

The invention provides a method for detecting the presence of an antigen from a cell capable of under-going rapid lysis to release the antigen into solution. The method includes mixing a sample solution with an aqueous diluent having an effective amount of an agglutination enhancer which contains a non-protein water soluble polymer and a water soluble protein. The solution of sample and agglutination enhancer is then contacted with an antibody specific for the antigen. Agglutination indicates the presence of the antigen.

AGGLUTINATION TEST

Field of the Invention

This invention relates to an enhanced antigen/antibody agglutination test.

Background of the Invention

Aggregates that quickly sediment can be formed from mixing an antigen and antibody in the presence of an electrolyte, for example, NaCl. Visible aggregates occur from mixing a suspension of particles with antigens on their surfaces with particles having a corresponding antibody. Such aggregation is termed agglutination.

Tests based upon the agglutination principle are generally technically simplistic. For example, a drop of a suspension of antigen particles and a drop of an antibody, usually serum containing the antibody, are mixed on a slide. The slide is rotated, by hand or by a machine, and in a few minutes agglutination occurs. Observing the slide with either the naked eye or under a microscope determines the presence or absence of agglutination. Agglutination is a positive test result.

Agglutination tests can also be performed in test tubes. The contents can be checked for agglutination while still in the tube or removed and examined more closely. A variation of the agglutination test involves the use of antibody-coated particles. In the tube variation, the antigen can be a soluble antigen, that is, sedimentation of the antigen is not required. The end result of a positive tube test is still seen as an agglutination, because the antibody-coated particles add the mass for the reaction to be seen. Thus, these larger antibody-coated particles result in a more sensitive test.

for enhancing antigen-antibody reactions in complex immunoassays (Siersted, et al., Methods of Enzymology 74 538 (1981)).

Istrate (PCT/US 92/01121 (1992)) describes an antigen extraction procedure and subsequent agglutination test for detecting an antigen specific for Mycobacterium tuberculosis when present in culture and in clinical material. The test uses monoclonal antibody-coated latex particles specific for detecting the extracted and concentrated lipoarabinomann (LAM) antigen. The test also requires the addition of a water soluble polymer to enhance the agglutination test. However, use of the water soluble polymer agglutination enhancer by Istrate did not provide sufficient sensitivity to avoid the need for concentration procedures.

The agglutination test of Istrate for the LAM antigen of Mycobacterium tuberculosis from culture requires a series of extraction and concentration steps. These steps include sonication and acetone precipitation of the extracted antigen-containing preparation. Absent extraction and concentration, the assay of Istrate is not sensitive enough to detect the antigen at clinical sample concentration levels.

As illustrated by Istrate, when cells are present in clinical material and a target antigen is only present in or on the cell, sensitivity of the agglutination test is low. Frequently, sophisticated equipment is required to isolate cells and concentrate an antigen before testing.

Accordingly, there is a need for improved agglutination test reagents. Also, a practical aggluti-nation test is needed which is sensitive enough to detect antigens at lower clinical concentrations without the need for an antigen concentration step.

Summary of the Invention

A method for detecting the presence of a target cell capable of undergoing rapid lysis comprising the following steps:

(a) obtaining a biological sample to be tested for the presence of the target cell which expresses a selected target antigen;

(c) neutralizing the antigen solution with a strong acid to form a neutralized solution;

(d) contacting the neutralized solution with particles whose surfaces contain an antibody, which is specific for the selected target antigen, under conditions permitting agglutination of the particles, which agglutination is visible to the naked eye or under a microscope; and

(e) detecting agglutination of the particles as an indication of the presence of the target cell in the biological sample. In a preferred method the aqueous diluent further comprises from about 0.01% to about 1.0% by weight of a chelating agent based upon the weight of the aqueous diluent.

In a further embodiment the present invention provides an aqueous cell lysis reagent composition comprising:

(i) from about 1% to about 3% by weight of of a non-protein water soluble polymer, based upon the total composition weight;

(ii) from about 0.2% to about 2% by weight of a water soluble protein, based upon the total composition weight; and

(iii) from about 0.1% to about 4.0% by weight of a strong base, based upon the total composition weight.

A preferred aqueous cell lysis composition further comprises from about 0.01% to about 1.0% by weight of a chelating agent, based upon the total composition weight.

Preferably, in each of the above embodiments of the invention:

the non-protein water soluble polymer is selected from the group consisting of polyethylene glycol, dextran, or gum acacia;

the water soluble protein is gelatin or albumin;

the chelating agent is ethylenediaminetetra-acetic acid (EDTA); and

the strong base is a NaOH solution.

Lysis of target cells is obtained by exposing the cells to a strong base, followed by exposure to a strong acid which neutralizes the base and adjusts the pH. Ideally, lysis can be obtained using an aqueous diluent containing from about 0.2% to 0.5% by weight of NaOH and about 0.005 % phenol red as an indicator. The preferred neutralizing agent is HCl.

Preferably, the method of the invention is applied to any bacteria cells and to other cells, human or otherwise, which can be lysed by NaOH, or similar alkaline agents. Whether rapid lysis is possible can be quickly determined for a particular cell type by routine experimentation.

Cells which are readily lysed may include pathogenic bacteria from genera such as Hemophilus, Neisseria, Streptococcus, Staphylococcus, Escherichia, Clostridia, Pseudomonas, Proteus, and the like. Human cells are readily and rapidly chemically lysed.

Upon being chemically lysed the target cells release target antigens. The target antigens to be detected are those which are not altered by the alkaline lysis technique to such a degree as to be no longer capable of recognition of by a specific antibody in the presence of a water soluble polymer-water soluble protein-chelating agent mixture.

Whether a particular antigen is altered too much by the alkaline lysis conditions to be detected is readily determined as follows. Target cells are obtained from a cell culture or tissue culture in sufficient quantity to produce a 1 McFarland concentration in phosphate buffered saline. Mechanical and alkaline chemical lysing procedures are run on pairs of identical concentration test target cell samples to produce solutions which have the target antigen. The test samples are obtained from progressive 1/2 cell-concentration dilutions of the 1 McFarland stock cell solution. These dilutions are obtained by diluting a volume of solution having a particular concentration of target cells with an equal volume of buffer solution. Thus, the cell concentration per unit volume is halved.

Agglutination assays of the resulting antigen solutions from each of the pairs of solutions are compared

proteins obtained by hydrolysis of collagen by boiling skin, ligaments, tendons, etc. Gelatin is strongly hydrophilic, absorbing up to ten times its weight in water. Albumin is any one of a group of water-soluble proteins of wide occurrence in such natural products as milk (lactalbumin), blood serum, and eggs (ovalbumin).

The combination of a non-protein water-soluble polymer enhancer and a water soluble protein enhancer permits a very sensitive agglutination test. Surpris-ingly, at the enhancer concentrations set forth above for the aqueous diluent, autoagglutination is avoided while providing sufficient enhancement to permit detection of antigens at clinical concentrations. Thus, the need for an antigen concentration step is avoided or minimized.

In agglutination tests with lysates made in a non-protein water-soluble polymer and a protein water soluble polymer diluent solution, DNA from the lysates can act as a further agglutination enhancer. Agglutination enhancement due to DNA can be optimized by adding a chelating agent to the lysate prior to testing. An example of an acceptable chelating agent is EDTA, which is known to chelate ions necessary for the action of deoxyribonucleases (DNases). Thus, EDTA in the lysate can preserve DNA enhancement of the agglutination test by neutralizing the action of DNases. The enhancing effect of DNA can be eliminated by adding and mixing DNase to a solution on an agglutination slide. About five microliters of a 2000 mg/ml solution of DNase per slide is sufficient.

A preferred diluent solution contains from about 1% to 2% by weight of PEG, from about 0.25% to about 1% by weight of gelatin, and from about 0.03% to about 0.40% by weight of EDTA.

PEG having a molecular weight from about 200 to about 15,000 is acceptable for the diluent solution. About 8000 is the preferred molecular weight for the PEG polymer, but PEG 350 and PEG 15,000 can be substituted. PEG having a molecular weight less than 200 may have insufficient viscosity. PEG having a molecular weight more than 15,000 can be more difficult to work with due to excessive viscosity.

Rhone Poulenc, France. Such beads provide a desirable contrast against an opaque slide that is white.

The particles are coated with antibodies specific for the selected target antigen, that is, an antigen characteristic of the target cell or organism being detected. The surface of a particle may be coated using known methods capable of directly or indirectly attaching antibodies. The antibodies may be absorbed directly on the surface of the particle or attached to the particle through a spacer molecule, e.g., a molecule capable of bonding to both the surface of the particle and to the antibody. Preferably, the antibodies are directly attached or absorbed to the particle using pas-sive coating techniques well-known to those of ordinary skill in the art. Such coating techniques tend to pre-serve the specificity and activity of the immunological reagent.

The term antibody is intended to include whole polyclonal or monoclonal antibodies, antibody fragments such as Fab fragments, chimeric antibodies containing portions from two different species, and synthetic peptides identical to or functionally analogous to the antibody. The preferred form of antibody is whole, monoclonal antibody. It should be understood that more than one species of monoclonal antibody may be attached to a particle. Examples of monoclonal antibody-producing cell lines include hybridoma cell lines, myeloma cell lines, or viral or ontogenically transformed lymphoid cells. Hybridoma cells which can produce the specific antibodies for use with the present invention may be made by the standard somatic cell hybridization technique of Kohler and Milstein, Nature 256 495 (1975) or similar procedures employing different fusing agents.

The agglutination conditions according to the invention are described generally as follows.

The conditions and reagents are preferred which permit visually detectable agglutination to occur. The naked eye can detect an agglutination clump when it is about 50μ in size. Thus, visually detectable aggluti- nation requires approximately 100 antibody-coated par- ticles, with each particle being about 0.2μ in diameter, to be attached together

For example, 0.5 ml of the susp[illegible]sion is added to 0.5 ml of PBS and the resulting 1 ml sol[illegible]on is thoroughly mixed to produce a 1:2 dilution solution The 1:2 dilution solution has 1/2 the concentration of cells per volume as compared to the 1 McFarland cell suspension solution. This 1:2 dilution solution can be further progressively diluted two-fold in a similar manner with PBS to produce a series of two-fold dilution solutions, that have cell concentrations of 1/4, 1/8, 1/16, etc., as compared to the original 1 McFarland cell suspension solution. This dilution procedure allows one to determine the minimum concentration of cells in solution which, when lysed, will result in a positive agglutination test result.

After forming the progressive dilution solutions, the cells of each solution are lysed with a strong base to produce a lysed cell solution. About 50 μl of a strong base such as 1N NaOH solution per 0.5 ml of cellular solution is usually adequate to rapidly lyse the cells. However, the concentration of strong base may be varied for a particular cell type. After lysing the cells the pH of the lysed cell solution should be returned to neutrality. The neutralization process is best controlled by monitoring the pH. An electronic pH meter can be used to determine the neutrality point or a pH indicator can be used to visually indicate it. About 0.005 % by weight of a pH indicator such as phenol red can be added to sample prior to adding a pH neutralizing acid. The phenol red pH indicator provides a color change which readily indicates the neutrality point and avoids the need for an expensive pH meter. The amount of strong acid such as 1N HCl needed to neutralize the alkaline extract can then be judged by the color change. Usually the amount of strong acid required is less than 50 μl per 0.5 ml of cellular solution. the exact amount in any particular situation can be determined by adding acid to the negative control, which is void of bacteria cells.

For the agglutination reaction, about 50 μl of lysate and about 25 μl of the reagent, which is antibody-coated particles, can be put in the circle of a glass agglutination slide, and mixed with an applicator stick. The slide

identified. Using a wood applicator stick a colony can be touched and transferred to about 0.5 ml of the PEG-gelatin-EDTA mixture and lysed according to the method of this invention. When the lysate is mixed with the specific monoclonal antibody-particles in an agglutination test, a positive test result is obtained.

The following non-limited examples are provided to illustrate the invention.

Examples

EXAMPLE 1 : Agglutination of Cultured N. Gonorrhoeae

A. Preparing the Aqueous Diluent

A 2X stock aqueous diluent of polyethylene glycol, gelatin and EDTA in phosphate buffered saline are prepared as follows. The 2X stock is diluted in half with phosphate buffered saline for use in the aggluti-nation test.

Phosphate buffered saline (PBS) was made by adding one part of one molar (1M) sodium phosphate buffer (pH 7) to thirty-nine parts of 0.85% (w/v) saline. Gel-atin was prepared as a 10% solution (w/v) in distilled water, and autoclaved. The gelatin was liquified by heating in a warm water bath. To 60 ml of PBS, was added 0.6 grams of EDTA and 0.8 ml of 1N NaOH. Heating in a warm water bath speeded up the incorporation of EDTA in the liquid. Eight ml of the 10% gelatin was then added, followed by 3.2 grams of PEG 8000 (Sigma Chemical Co., St. Louis, MO). The volume of the solution was adjusted to 80 ml by adding PBS.

B. Monoclonal Antibody-coated Particles

Monoclonal antibody-coated staphylococcus reagent W1 was obtained from Karo-Bio, Sweden, distributed as "Phadebact Gonococcal Reagent." An agglutination reagent solution was formed by diluting the W1 reagent 1:5 in PBS.

C. Preparation of Lysate

N. gonorrhoeae of serogroup W1, National Reference Laboratory, U.S.A., strain 32779, was grown on solid media

The results were the essentially the same for the mechanically rotated and hand rotated slides, both of which are standard methods used in agglutination tests. The slides were evaluated for the presence of agglutination both by eye and under a 10X power micro-scope. The results are set forth below in Table 1.

Table 1

N. Gonorrhoeae Culture Agglutination Results

Cell Suspension Concentration	Agglutination Visible by Eye	Agglutination Visible at 10X
1:1 McFarland	+	+
1:2	+	+
1:4	+	+
1:8	+	+
1:16	+	+
1:32	+	+
1:64	+	+
1:128	+	+
1:256	-	+
1:512	-	+/-
0 (Control)	-	-

\+ = visible agglutination
\- = no visible agglutination

The lysate from the cell solutions with the greatest concentration of N. gonorrhoeae cells, that is, from the 1:1, 1:2, and 1:4 cell solutions, agglutinate in a minute or so. The greater dilutions, 1:32 and 1:64, take 10 to 20 minutes to develop into a visible agglutination.

When a mechanical rotator was used, weak agglutination reactions become stronger, if the slide was finally hand-rotated for a minute or two. The end point agglutination of the N. gonorrhoeae lysate in PEG-gelatin-EDTA was 1:128 as seen by the naked eye, and about two to four-fold higher as seen under a 10X microscope.

EXAMPLE 2 : Agglutination of Gonorrheal Clinical Sample

A. Collecting the Clinical Sample

Applicability of the invention to clinical material was demonstrated with five urethral swabs of pus taken from

All references cited with respect to synthetic, preparative and analytical procedures are incorporated herein by reference.

The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

gelatin or albumin based upon the weight of said aqueous diluent; and

(iii) from about 0.01% to about 1.0% by weight of a chelating agent based upon the weight of said aqueous diluent.

3. A method according to claim 2, wherein said buffered solution is a buffered saline solution.

4. A method according to claim 3, wherein said aqueous diluent comprises:

(i) from about 1% to about 3% by weight of polyethylene glycol;

(ii) from about 0.2% to about 2% by weight of gelatin;
and

(iii) from about 0.03% to about 0.40% of ethylenediaminetetraacetic acid.

5. A method according to claim 4, wherein said aqueous diluent comprises:

(i) from about 1% to about 2% by weight of polyethylene glycol;

(ii) from about 0.25% to about 1% by weight of gelatin;
and

(iii) from about 0.03% to about 0.40% of ethylenediaminetetraacetic acid.

6. A method according to claim 4, wherein said agglutination is visible to the naked eye.

7. A method according to claim 1, wherein the amount of strong base is from about 0.1% to about 4.0% by weight based upon the weight of said aqueous diluent.

8. A method according to claim 1, wherein the amount of strong base is from about 0.2% to about 0.5% by weight based upon the weight of said aqueous diluent.

18. A method according to claim 1, wherein said antigen is a membrane-bound antigen.

19. A method according to claim 1, wherein said process can be completed in about fifteen minutes or less.

20. An aqueous cell lysis reagent composition comprising:

(i) from about 1% to about 3% by weight of of a non-protein water soluble polymer, based upon the total composition weight;

(ii) from about 0.2% to about 2% by weight of a water soluble protein, based upon the total composition weight;

(iii) from about 0.01% to about 1.0% by weight of a chelating agent, based upon the total composition weight; and

(iv) from about 0.1% to about 4.0% by weight of a strong base, based upon the total composition weight.

21. A composition according to claim 20, which comprises a buffered solution containing:

(i) from about 1% to about 3% by weight of polyethylene glycol or dextran, based upon the weight of said composition;

(ii) from about 0.2% to about 2% by weight of gelatin or albumin, based upon the weight of said composition;

(iii) from about 0.01% to about 1.0% by weight of a chelating agent, based upon the weight of said composition; and

(iv) from about 0.1% to about 4.0% by weight of a strong base, based upon the weight of said composition.

22. A composition according to claim 21, wherein said aqueous reagent composition is a buffered saline solution.

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/571
US CL : 435/7.36

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	"MICROPARTICLE IMMUNOASSAY TECHNIQUES" published 1988 by Seradyn, Inc. (Indianapolis, IN), pages 1-49, see page 5, Slide Agglutination, page 12, last paragraph, page 29, page 34, pages 39-40.	1-29
Y	US, A, 4,362,531 (DE STEENWINKEL ET AL.) 07 December 1982, see especially column 3, lines 45-61 and column 4, lines 20-32.	1-29
Y	US, A, 4,497,900 (ABRAM ET AL.) 05 February 1985, see especially column 2, lines 43-61.	1-29
Y	US, A, 4,916,057 (THOMPSON ET AL.) 10 April 1990, see especially column 1, lines 42-59 and column 3, lines 56-60.	1-29

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search	Date of mailing of the international search report
27 DECEMBER 1994	03.02.95
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INTERNATIONAL SEARCH REPORT

International application No.
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B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

435/7.2, 7.21, 7.22, 7.23, 7.24, 7.25, 7.3, 7.31, 7.32, 7.33, 7.34, 7.35, 7.36, 7.37, 29, 961, 962, 967; 436/17, 18, 518, 534, 174, 175, 176, 825, 826

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG
search terms: PEG or polyethylene glycol; gelatin; EDTA or ethylenediaminetetraacetic acid; Zubrzycki, Leonard J.; agglutinat?; neisseria; dextran; gelatin? ?; albumin; EDTA; diluent; CD cells

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